The emergence of a new strain of porcine circovirus-2 in Ontario and Quebec swine and its association with severe porcine circovirus associated disease — 2004–2006

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Abstract

In the late fall of 2004 more severe lesions of porcine circovirus-2 associated disease (PCVAD) than usual occurred during an outbreak of porcine circovirus-2 (PCV-2) infection in Ontario nursery and grower/finisher pigs. The lesions were of unprecedented severity and included diffuse bronchointerstitial pneumonia, granulomatous enteritis, vasculitis, interstitial nephritis, and new lesions of splenic infarction. Some affected herds had up to 50% mortality. The outbreak correlated with the sudden emergence of a variant PCV-2, with PCR restriction fragment length polymorphism (RFLP) type 321. Phylogenetic comparison of ORF2 sequences and full genome sequences showed the new variant to be different from the previously dominant RFLP type 422 viruses, and similar to viruses that had occurred in France and other European and Asian countries. A subsequent retrospective study showed a statistically significant increase in the frequency of histological lesions in lymph node, spleen, lung, small intestine, colon and kidney, for pigs spontaneously infected with RFLP type 321, compared with the older RFLP type 422 strain. Viral burden, based on IHC staining in lymph node, also showed a statistically significant increase in pigs infected with the newer variant RFLP type 321, compared with the older RFLP type 422 strain. This enhanced virulence in pigs infected with PCV-2 RFLP type 321 strain may be related to the genetic differences in this new strain of PCV-2. This virus is now the dominant strain of PCV-2 virus found in Ontario and Quebec swine.

Résumé

À l'automne 2004 des lésions plus sévères que d'habitude de maladie associée au circovirus porcin de type 2 (PCVAD) ont été observées au cours d'un épisode d'infection par le circovirus porcin de type 2 (PCV-2) chez des porcs en pouponnière et en croissance/finition en Ontario. Les lésions étaient d'une sévérité sans précédent et incluaient une pneumonie broncho-interstitielle diffuse, une entérite granulomateuse, une vasculite, une néphrite interstitielle, et de nouvelles lésions d'infarcti spléniques. Quelques un des troupeaux affectés présentaient jusqu'à 50 % de mortalité. L'épisode corrélait avec l'émergence soudaine d'un variant de PCV-2, possédant un polymorphisme des fragments de restriction (RFLP) de type 321. Les comparaisons phylogénétiques des séquences ORF2 et des séquences du génome entier ont montré que le nouveau variant était différent des virus avec un RFLP de type 422 qui dominaient précédemment, et similaires aux virus s'étant manifestés en France et autres pays européens et asiatiques. Une étude rétrospective subséquente a montré une augmentation statistiquement significative de la fréquence de lésions histologiques dans les nœuds lymphatiques, la rate, les poumons, le petit intestin, le côlon et les reins, pour les porcs infectés de manière spontanée par le virus de type RFLP 321, comparativement à la souche plus ancienne de type RFLP 422. La mesure de la charge virale, basée sur la coloration par IHC des nœuds lymphatiques, a également montré une augmentation statistiquement significative chez les porcs infectés avec le nouveau variant de type RFLP 321, comparativement à la souche plus ancienne RFLP type 422. Cette augmentation de virulence chez les porcs infectés avec le PCV-2 de type RFLP 321 pourrait être reliée aux différences génétiques dans cette nouvelle souche de PCV-type 2. Ce virus est maintenant la souche virale dominante de PCV-2 trouvée chez les porcs en Ontario et au Québec.

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Introduction

In 1996, reports from western Canada indicated the presence of a previously unrecognized syndrome in swine herds (1). The predominant clinical features of this postweaning multisystemic wasting syndrome (PMWS), now referred to as porcine circovirus associated disease (PCVAD), were unthriftiness in nursery and grower pigs, jaundice, weight loss, dyspnea, diarrhea, and lymphadenopathy, with interstitial pneumonia on necropsy. Histological lesions included lymphohistiocytic to granulomatous interstitial pneumonia, multinucleated giant cells, lymphoid depletion, and the presence of intracytoplasmic inclusion bodies in macrophages (2). The clinical expression now also includes porcine dermatitis and nephropathy (PDNS), reproductive failure, perinatal myocarditis, porcine respiratory disease complex, porcine necrotizing pneumonia (PNP), granulomatous enteritis, necrotizing lymphadenitis, and congenital tremors (3,4). The etiology of this condition was attributed to porcine circovirus (5–7). This has been designated as porcine circovirus-2 (PCV-2), to differentiate it from the nonpathogenic porcine circovirus-1 (PCV-1) (8).

Circoviruses are small (17 nm), icosahedral, nonenveloped viruses with a circular, single-stranded deoxyribonucleic acid (DNA) genome (9) classified in the genus *Circovirus* and the family *Circoviridae* (10). Two major open reading frames (ORF) are recognized, ORF1, which encodes proteins involved with viral replication, and ORF2 which encodes the capsid protein (11). The capsid protein has been shown to be a reliable phylogenetic marker for PCV-2 (12). A smaller ORF3, nonessential for viral replication, has been linked with viral pathogenesis and apoptosis in vivo using mice (13). Other viruses within this family include chicken anemia virus and psittacine beak and feather disease virus (10).

The factors triggering clinical expression of PCV-2 infection are poorly understood and there has been difficulty inducing disease experimentally with PCV-2 (4). Various experimental models have been developed including co-infection with other agents, such as porcine parvovirus (14–16), porcine reproductive and respiratory syndrome virus (PRRSV) (17–19), or *Mycoplasma hyopneumoniae* (20), in an attempt to explain the variable presentation of PCV-2 associated disease.

In the late fall of 2004 new and more severe lesions began to appear in Ontario nursery and grower/finisher pigs associated with PCV-2 infection, with pneumonia and diarrhea and up to 50% mortality in some affected swine herds. A similar disease occurred in Quebec swine at about the same time (21). The number of PCV-2 associated disease laboratory submissions to the Animal Health Laboratory (AHL), University of Guelph increased dramatically in Ontario beginning in December 2004 (Figure 1A). In 2005 there were 350 pathology cases (8.9% of all swine cases) associated with PCV-2 infection, in comparison with 60 cases (2.3% of all swine cases) in 2004. The outbreak continued into 2006 with 408 cases (10.1% of all swine cases). These cases were correlated with the sudden emergence in 2005 and subsequent dominance of a variant PCV-2 strain with polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) type 321 (Figure 1B) in Ontario swine.

This report describes the development of the PCV PCR and RFLP typing technique used, and the initial 2004–2006 Ontario

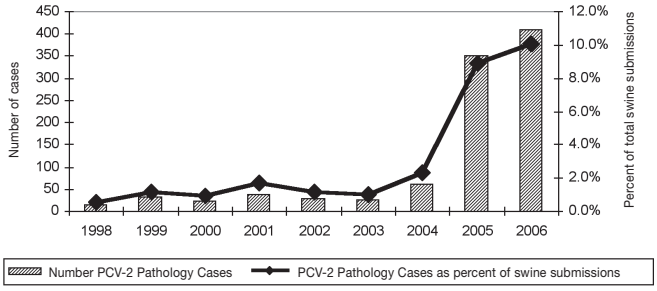

Figure 1A. Number of porcine circovirus-2 (PCV-2) pathology cases and percent of PCV-2 pathology cases of total swine submissions to the Animal Health Laboratory, University of Guelph, 1998 to 2006.

PCV-2 outbreak attributed to this emerging PCV-2 variant, which has been only briefly described previously (22–24). The outbreak prompted a retrospective study in order to further evaluate the perceived changes in the pathologic expression of PCVAD in Ontario swine. For this study, cases that were previously submitted to the AHL were selected for further comparison, including evaluation of histopathologic lesions, immunohistochemical (IHC) staining for PCV-2 antigen in lymph node, and PCR RFLP typing of PCV-2 from tissue. The study also included the evaluation of 10 cases from Quebec by IHC staining of lymph nodes. Partial ORF2 genome sequences were compared at the time of the outbreak. The entire genome was sequenced retrospectively for 3 representative Ontario RFLP type 422 viruses, 3 representative Ontario RFLP type 321 new variant viruses, and 10 Quebec PCV-2 viruses from similarly affected pigs. Sequences were compared to those in GenBank. http://www.psc.edu/general/software/packages/genbank/genbank.php

Materials and methods

DNA extraction

The DNeasy Tissue Kit (Qiagen, Mississauga, Ontario) was used as directed by the manufacturer to extract DNA from tissues.

PCR Analysis

Primers for initial diagnosis for all Ontario cases were selected using sequence information for both PCV-1 and PCV-2 genomes obtained from GenBank (Accession no. U49186 and AF027217). Primers to amplify both virus types were selected using Primer Designer Version 2.01 (1990, Scientific and Educational Software). The forward primer: 5′-GAA TGG TAC T (genomic map position 831 to 850) and the reverse primer: 5′-ACG TAT CCA AGG AGG CG TTA-3′ (genomic map position 1732 to 1713) amplified a 902 base pair (bp) product that represented ORF2 and part of ORF 1. This section of the viral genome was selected to subsequently distinquish PCV-1 from PCV-2 since the ORF2 was more polymorphic then ORF1 (25). The master mix was prepared in 50 µL final volume using 2.5 U Amplitaq Gold DNA polymerase (Applied Biosystems, Mississauga, Ontario), 1X PCR buffer II, 2 mM $MgCl_2$ (Applied Biosystems), 0.2 mM of each dNTP (Applied Biosystems)

Table I. Restriction fragment length polymorphism (RFLP) typing patterns for porcine circovirus showing the size of bands following digestion with each restriction enzyme and patterns associated with either porcine circovirus-1 (PCV-1) or porcine circovirus-2 (PCV-2)

Restriction enzyme	RFLP type	Size of bands (bp)
*Xba*I	1	no cut
	2	150, 750
	3	375, 525
	4	196, 332, 373
*Eco*RI	1	no cut
	2	311, 590
*Sma*I	1	no cut
	2	242, 659
PCV-1	RFLP types 111 or 211	
PCV-2	RFLP types 422, 322, 122, 321	

and 0.1 mM primers (Guelph Molecular Supercentre, Laboratory Services Division, University of Guelph, Guelph, Ontario). The sample template volume was 2 µL. Polymerase chain reactions were performed using the following parameters: 95°C for 12 min, followed by 35 cycles of 95°C for 20 s, 60°C for 15 s, and 72°C for 20 s, with final extension at 72°C for 7 min. The PCR product was run on a 1.5% agarose gel (Canadian Life Technologies, Burlington, Ontario), stained with ethidium bromide, and the bands measured. The 902 bp products were initially sequenced to ensure that the PCR reaction was specific for PCV.

Restriction endonuclease digestion

A restriction fragment length polymorphism (RFLP) assay was developed to rapidly perform preliminary characterization and differentiate PCV-1 from PCV-2. The RFLP types were differentiated using restriction enzymes (RE) predicted to discriminate between PCV-1 and PCV-2 and between multiple strains of PCV-2, based on comparing multiple sequence alignments for all PCV-1 and PCV-2 sequences found in GenBank at the time of test development in 1998. Digestion of 10 µL of each PCR product with each of the enzymes *Xba*I, *Eco*RI and *Sma*I (Amersham Biosciences, Piscalaway, New Jersey, USA) was done at 37°C for 2 h in the buffer supplied by the manufacturer. Digested PCR products were analyzed alongside the undigested product in a 2% NuSieve agarose gel (BioWhittaker Molecular Applications, Rockland, Maine, USA), stained with ethidium bromide and the bands sizes measured by comparing to size markers (100 bp DNA ladder; Invitrogen Canada, Burlington, Ontario). The RFLP types were designated as listed in Table I. GenBank PCV-2 sequences from North American isolates produced 3 different predicted RFLP cleavage patterns (RFLP type 422, 322, 122, with the 1st digit referring to the RFLP pattern for *Xba*I, the 2nd digit referring to the RFLP type pattern for *Eco*RI, and the 3rd digit referring to the RFLP pattern for *Sma*I), differing only at the *Xba*I site, while a pattern from a French viral isolate differed in both the *Xba*I and the *Sma*I sites (RFLP type 321).

Sequencing

Automated sequencing of ORF2 PCR products for PCVAD outbreak RFLP type 321 cases and an earlier RFLP type 422 strain was performed at the Guelph Molecular Supercentre on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Mississauga, Ontario) using the primers previously described. Sequence alignments were performed using the multiple alignment program Clustal W (DNA Star versiom 6; MegAlign, Madison, Wisconsin, USA). Full length genome sequences were prepared retrospectively for 3 representative PCV-2 RFLP type 422 strains and 3 representative PCV-2 RFLP type 321 new variant strains from Ontario cases included in the retrospective study, and 10 Quebec strains representative of the 10 cases included in the IHC staining for lymph node. Full-length genome sequencing was as previously described (26), using 2 overlapping PCR products. The resulting complete genome sequences were compared to PCV-2 sequences available on GenBank, using the Clustal W alignment method from the BioEdit Sequence Alignment Editor (version 7.0.5.2) software.

Case selection for retrospective study

Swine submissions to the AHL from years 2001 through 2005 were searched retrospectively in the computerized reporting system to identify those with PCV-2 noted in diagnostic codes. Case criteria for inclusion in this study were positive PCV-2 status identified by PCR analysis of tissue (typically lung and tonsil) with subsequent PCR RFLP typing, and submission of formalin-fixed lung, lymph node, spleen, small intestine, and colon for histopathology. Both in-house necropsy cases and "mail-in" cases, with necropsies conducted by submitting veterinarians, were included in the study. The AHL cases meeting all criteria for inclusion in the study included 11 cases from 11 different herds (28 animals) with identification of RFLP type 422 PCV-2, and 15 cases from 14 herds (44 animals) with RFLP type 321 PCV-2. In addition, 10 cases that were submitted to the Laboratoire d'expertise en pathologie animale du Québec, Sainte-Foy, Québec,

Table II. Tissue-specific histopathologic lesions evaluated in porcine circovirus-2 (PCV-2) PCR restriction fragment length polymorphism types 422 and 321 infected pigs included in the retrospective study scored blindly as present or absent by 2 pathologists

Lymph node/spleen	Lung	Small intestine/colon	Kidney
Depletion of lymphoid follicles	Consolidation	Infiltration of lamina propria by histiocytes and lymphocytes	Multifocal cortical interstitial aggregates of histiocytes and lymphocytes
Histiocytic infiltration	Interlobular edema	Infiltration of submucosa by histiocytes and lymphocytes	Fibrinous glomerulitis
PCV-2-type inclusions	Subpleural edema	Submucosal lymphoid atrophy and histiocytic replacement	PCV-2-type inclusions
Giant cells	Perivascular/peribronchiolar lymphohistiocytic cuffing	PCV-2-type inclusions	Giant cells
Vasculitis	Perivascular/peribronchiolar fibrosis	Giant cells	Vasculitis
Fibrin exudation	BALT depletion	Perivascular lymphocytic cuffing in tunica muscularis	
Consolidation	Bronchiolar epithelial necrosis		
	Alveolar septal thickening		
	Alveolar macrophages		
	Giant cells		
	PCV-2-type inclusions		
	Secondary lesions: – Suppurative bronchopneumonia – Fibrinosuppurative bronchopneumonia – Hemorrhage – Intravascular thrombosis		

with similar disease and in which PCV-2 was identified, were also included in the study.

Histopathology

Tissues were fixed in buffered formalin at the time of necropsy, processed and sectioned according to standard protocols, and stained with hematoxylin and eosin. For the retrospective study, 2 pathologists from the AHL conducted blinded histopathologic evaluation, which was limited to the tissues previously listed; kidney was also examined in cases where it was available. Tissues were evaluated for presence or absence of the specific lesions listed in Table II.

Immunohistochemistry

Automated IHC procedures were performed on multiple tissues for PCVAD outbreak cases. However for the retrospective study, only lymph nodes were evaluated and staining was performed in 4 separate batches to include samples from 23 Ontario pigs infected with PCV-2 RFLP type 422, 42 Ontario pigs infected with PCV-2 RFLP type 321, and 10 Quebec pigs infected with the new variant virus. For both outbreak cases and retrospective cases staining was performed using a DAKO autostainer (DakoCytomation, Mississauga, Ontario). Processed 4-μm sections from formalin-fixed, paraffin-embedded lymph node were deparaffinized and rehydrated, treated with 3% hydrogen peroxide for 10 min to block endogenous tissue peroxidases, and subsequently treated with Proteinase K (DakoCytomation) for 12 min at room temperature (RT). Following a 10-min incubation with universal blocker (DakoCytomation), tissue sections were incubated with anti-PCV-2 rabbit polyclonal antiserum (Dr. P. Halbur, Iowa State University, Ames, Iowa, USA) at a dilution of 1:2000 for 60 min at RT. A peroxidase-labeled streptavidin-biotin visualization kit (LASB 2; DakoCytomation) was used following the manufacturer's recommendations, and Nova Red (Vector Laboratories Canada, Burlington, Ontario) was applied as chromogen. Tissues were counterstained with Harris Hematoxylin (Fisher Scientific, Toronto, Ontario). As negative controls for each tissue, nonimmune rabbit serum, diluted to a protein concentration similar to that of the PCV-2 antiserum, was substituted for primary antibody. Tissue sections from the same positive control block were included in each run and assessed microscopically for consistency of IHC staining intensity and distribution between batches. For the retrospective study, stained slides were examined by one pathologist who assigned a score from 0 to 5, based on staining distribution in a single lymph node from each pig stained with anti-PCV-2 rabbit polyclonal antiserum and scored as follows: 0 = no staining; 1 = scattered individual antigen-positive mononuclear cells (histiocytes); 2 = scattered individual antigen-positive mononuclear cells (histiocytes) and low numbers of focal aggregates of antigen-positive cells, with involvement of < 20% of node parenchyma; 3 = multiple foci of antigen-positive cellular aggregates, involving 20% to 50% of node parenchyma; 4 = confluent foci of antigen-positive cellular

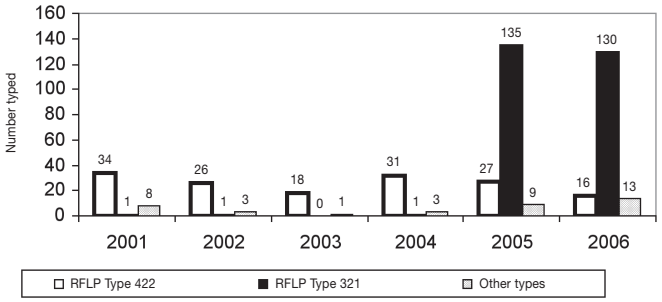

Figure 1B. PCR-RFLP typing patterns of porcine circovirus-2 (PCV-2) strains using *Xba*I, *Eco*RI, and *Sma*I restriction enzymes for cases submitted to the Animal Health Laboratory, University of Guelph, 2001 to 2006.

aggregates, involving > 50% of node parenchyma; modified from a previously published scoring system (27).

Statistical analysis

For the retrospective study, a Fisher's exact test was used to determine if there were statistically significant differences between the frequency of observed histological lesions associated with older PCV-2 RFLP type 422 strains versus the newer PCV-2 RFLP type 321 strains. Statistical significance was set at $P < 0.05$. An odds ratio (OR) was used to determine the strength of association of each lesion with PCV-2 RFLP type, for those in which statistically significant differences were found by the Fisher's exact test. A generalized linear mixed model was used to determine if there was statistically significant interaction between the pathologist and the PCV-2 RFLP type by case. A Wilcoxon two-sample test was used to determine if there was a statistically significant difference between the scores for IHC staining of the PCV-2 RFLP types. Statistical analyses were performed using SAS version® 9.1.3 (SAS Institute, Cary, North Carolina, USA).

Results

Restriction endonuclease analysis

Following PCR the expected product size of 902 bp was observed from tissue samples and sera of PCV-2 infected pigs. All positive samples were digested with the 3 RE (*Xba*I, *Eco*RI, and *Sma*I). Prior to December 2004, most cases demonstrated the expected RFLP type 422 cleavage pattern associated with North American strains of PCV-2. Occasional cases had RFLP type 421, identifying the loss of a restriction site to *Sma*I. After December 2004, a marked change occurred in the dominant RFLP type from the RFLP type 422 seen in previous years, to RFLP type 321 (Figure 1B), as a result of changes in *Xba*I and *Sma*I restriction sites. The number of cases with RFLP type 321 increased from 1 in 2004 to 135 in 2005, and 130 in 2006.

Sequencing

Sequence analysis of the ORF2 and partial ORF1 gene segment for outbreak cases showed the Ontario RFLP type 321 viruses to have > 99% sequence homology with each other, and 98% sequence homology with those reported for France, the United Kingdom,

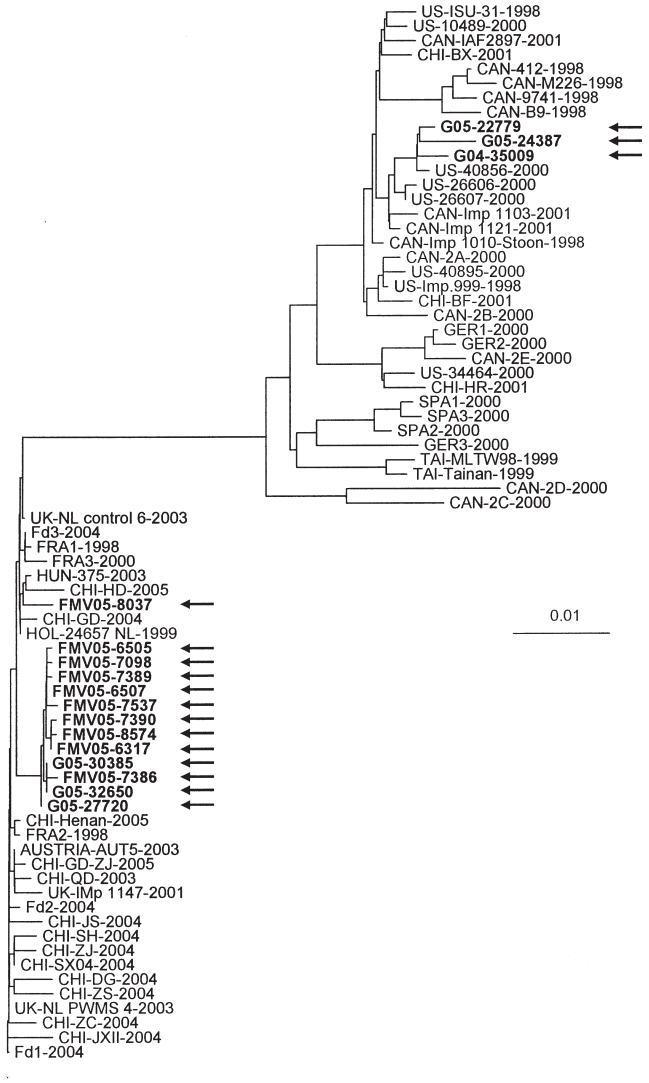

Figure 2. Dendrogram comparing the full genome sequences of porcine circovirus-2 (PCV-2) Ontario PCR restriction fragment length polymorphism (RFLP) type 422 viruses (G05-22779, G05-24387, G04-35009) and the new variant PCV-2 Ontario RFLP type 321 viruses (G05-30385, G05-32650, G05-27720, and Quebec viruses FMV05-6505, FMV-7098, FMV05-7389, FMV-6507, FMV05-7537, FMV05-7390, FMV05-8574, FMV05-6317, FMV05-7386, (all the above bolded and denoted by arrows) with PCV-2 strains available in GenBank, with year of recovery in order as they appear in Table III. Bar represents 0.01 or 1% DNA sequence divergence.

and China (data not shown). However these emerging Ontario RFLP type 321 viruses had only 91.6% sequence homology to the previously dominant Ontario RFLP type 422 viruses, and have only 92% to 93% sequence homology to those previously reported from the USA before 2005. The dendrogram comparing the full genome sequences prepared retrospectively for 3 Ontario PCV-2 RFLP type 422 viruses, 3 PCV-2 RFLP type 321 and 10 recent Quebec PCV-2 strains with sequences found in GenBank is presented in Figure 2. The geographic location and GenBank accession numbers for sequences in this comparison are summarized in Table III. The 3 RFLP type 422 viruses from Ontario (G05-22779, G05-24387, G04-35009) grouped separately from the 3 PCV-2 RFLP type 321 new variant strains from Ontario (G05-30385, G05-32650, G05-27720)

Table III. Identification of porcine circovirus-2 (PCV-2) strains used in the dendrogram for comparison of PCV-2 sequences from Ontario and Quebec swine (bolded) with those in GenBank in the order they appear in Figure 2

Geographic origin	Isolate identification	GenBank accession number	Geographic origin	Isolate identification	GenBank accession number
United States/Iowa	ISU-31	AJ223185	France	Fd3	AY321984
United States/Illinois	10489	AF264040	France	Imp. 1011-48121 (FRA1)	AF055393
Canada/Québec	IAF2897	AF408635	France	FRA3	AF201311
China	BX	AF381177	Hungrary	375	AY256460
Canada	412	AF085695	China	HD	AY916791
Canada	M226	AF086836	Canada/Québec	**FMV-05-8037**	DQ220738
Canada	9741	AF086835	China	GD	AY613854
Canada	B9	AF086834	Netherlands	24657 NL	AF201897
Canada/Ontario	**G05-22779**	EF394775	Canada/Québec	**FMV-05-6505**	DQ220729
Canada/Ontario	**G05-24387**	EF394776	Canada/Québec	**FMV-05-7098**	DQ220731
Canada/Ontario	**G04-35009**	EF394774	Canada/Québec	**FMV-05-7389**	DQ220734
United States/Missouri	40856	AF264041	Canada/Québec	**FMV-05-6507**	DQ220730
United States/Utah	26606	AF264038	Canada/Québec	**FMV-05-7537**	DQ220736
United States/Utah	26607	AF264039	Canada/Québec	**FMV-05-7390**	DQ220735
Canada/Alberta	Imp. 1103	AJ293867	Canada/Québec	**FMV-05-8574**	DQ220727
Canada/Saskatchewan	Imp. 1121	AJ293868	Canada/Québec	**FMV-05-6317**	DQ220728
Canada	Imp. 1010-Stoon	AF055392	Canada/Ontario	**G05-30385**	EF394778
Canada	2A	AF027217	Canada/Québec	**FMV-05-7386**	DQ220732
United States/Iowa	40895	AF264042	Canada/Ontario	**G05-32650**	EF394779
United States	Imp. 999	AF055391	Canada/Ontario	**G05-27720**	EF394777
China	BF	AF381175	China	Henan	AY969004
Canada	2B	AF112862	France	Imp. 1011-48285 (FRA2)	AF055394
Germany	GER1	AF201305	Austria	AUT5	AY424405
Germany	GER2	AF201306	China	GD-ZJ	DQ017036
Canada	2E	AF109399	China	QD	AY291316
Canada	34464	AF264043	UK	Imp. 1147	AJ293869
China	HR	AF381176	France	Fd2	AY321999
Spain	SPA1	AF201308	China	JS	AY691679
Spain	SPA3	AF201310	China	SH	AY291318
Spain	SPA2	AF201309	China	ZJ	AY686764
Germany	GER3	AF201307	China	SX04	AY604430
Taiwan	MLTW98 (TA1)	AF154679	China	DG	AY682993
Taiwan	Tainan (TA2)	AF166528	China	ZS	AY596823
Canada	2D	AF117753	UK	NL PMWS 4	AY484416
Canada	2C	AF109398	China	ZC	AY682997
UK	NL control 6	AY484412	China	JXII	AY732494
			France	Fd1	AY322000

and the 10 Quebec FMV05 new variant strains are all noted by arrows. The RFLP type 422 strains were similar to earlier North American strains. The RFLP type 321 variant viruses were similar to strains from France (FRA1-1998, FRA3-2000, Fd1-2004, Fd2-2004, and Fd3-2004) with 99.3% to 99.8% sequence homology amoung the viruses.

Histopathology

The PCVAD outbreak that began in December 2004. Clinically affected pigs had a rasping cough and/or diarrhea, and were in poor body condition. At necropsy, pigs with respiratory signs often had severe pulmonary edema. Histologically, there was diffuse bronchointerstitial pneumonia, with interlobular septa and subpleural connective tissue widely expanded by edema and with dilation and thrombosis of lymphatics. Alveoli were flooded by proteinaceous fluid often containing free-floating macrophages and lymphocytes. Mild peribronchial and perivascular lymphohistiocytic cuffing, with infiltration of mononuclear inflammatory cells into alveolar septa, was generally much less pronounced than in previous PCV-2 cases. Spleen and tonsil were also frequently affected with acute lymphoid necrosis. Although present in most cases, in some submissions laboratory tests failed to identify the presence of other pulmonary pathogens such as PRRSV, swine influenza virus, *Mycoplasma hyopneumoniae*, and *Streptococcus suis*.

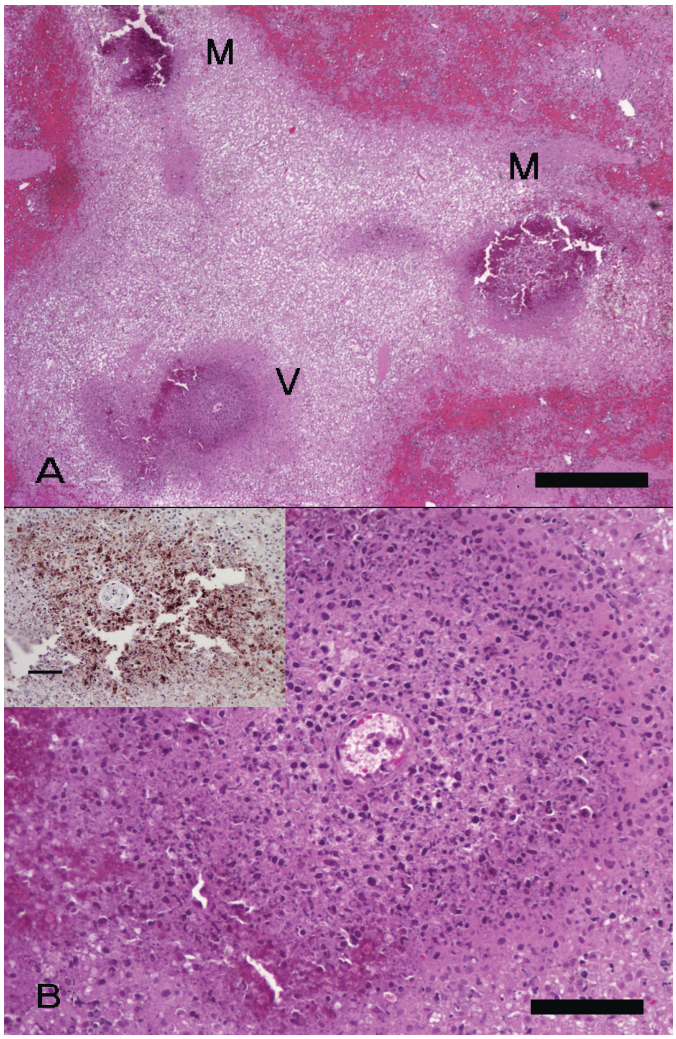

Figure 3. (A) Microscopic appearance of a splenic infarct associated with porcine circovirus-2 (PCV-2). Areas of mineralization (M) and vasculitis (V). Bar = 328 µm; (B) Higher magnification of blood vessel with vasculitis illustrating vascular necrosis and adjacent mononuclear cells; (B) Inset — Positive immunohistochemical staining for PCV-2 antigen of mononuclear cells surrounding this vessel. Bars = 50 µm.

In addition, there was gross enterocolitis with diarrhea in grower and finisher pigs that also was associated with PCV-2. There was thickening of intestinal walls in jejunum, ileum, and colon, reminiscent of *Lawsonia intracellularis*-induced porcine proliferative enteritis. Histologically, these animals had granulomatous enteritis and colitis, with lymphocytes in the villus mucosa, and extensive infiltration of histiocytes, lymphocytes, plasma cells and multinucleated giant cells throughout the lamina propria and the submucosa. In lymph node and submucosal follicles there was lymphocytolysis, with lymphoid depletion and sinusoidal histiocytic infiltration, more severe and extensive than previously seen. There were often numerous typical circoviral inclusions in both histiocytes and multinucleated giant cells.

Concurrently, there was an increase in cases of PCV-2 associated vasculitis and immune-complex disease, with lesions in kidney, lymph node, and spleen (Figure 3). In these cases, glomerulonephritis was consistently present, and sometimes animals had splenic infarcts evident both grossly and histologically, making it necessary to consider classical swine fever (CSF) as a differential diagnosis. At the beginning of the outbreak these samples were confirmed negative for CSF by the National Foreign Animal Disease Laboratory, Canadian Food Inspection Agency (Winnipeg, Manitoba). Cutaneous lesions were not evident in affected animals.

Retrospective study. Histological lesions of lymph node lymphocyte depletion, histiocytic infiltration, multinucleated giant cells and other lesions, as noted in Table IV, were more frequently identified in cases positive for the newer PCV-2 RFLP type 321 variant, versus the older RFLP type 422 strain ($P < 0.05$). The odds ratios and 95% confidence intervals (CI) indicating the strength of the association of the presence of specific lesions with the PCV-2 RFLP type 321 are summarized in Table IV. Pigs with RFLP type 321 infection, for example, were 9.33 times more likely to have lymphoid depletion of the lymph node than pigs with RFLP type 422 infection. The wide variation in 95% CI illustrates variability amoung animals and is influenced by sample size. For the lesions listed in Table II, there was no statistical interaction between the pathologist and the PCV-2 RFLP type by case.

Immunohistochemistry

The PCVAD outbreak that began in December 2004. Using IHC staining, outbreak cases had subjectively much greater PCV-2 antigen loads in lymph node, lung, small intestine, and large intestine than seen prior to the fall of 2004. Immunohistochemical staining of intestine showed circovirus antigen in macrophages in the lamina propria and submucosa. Using IHC, PCV-2 antigen was rarely found in association with vascular lesions, although variable amounts of antigen were present in other tissues including lung. In several cases with clinical neurological disease, PCV-2 antigen was demonstrated in association with endothelial or inflammatory cells in brain, as well as in other tissues.

Retrospective study. Immunohistochemical staining of lymph node sections demonstrated higher PCV-2 antigen load in pigs infected with the RFLP type 321 variant virus, versus the previously dominant RFLP type 422 virus. Among RFLP type 321-infected pigs, 64% had IHC scores of 3 and 4, whereas 65% of RFLP type 422-infected pigs were scored as 0, 1, or 2. This difference was statistically significant ($P = 0.0182$), with increased quantities of staining in lymph nodes from pigs from Ontario and Quebec infected with the newer variant RFLP type 321.

Discussion

The PCR RFLP typing method was initially developed to distinguish between PCV-1 and PCV-2, as was another PCR RFLP typing method (28). Routine use of the PCR RFLP typing method reported here proved useful in identifying the emergence of a new RFLP type 321 variant of PCV-2 that was introduced into Ontario swine herds in December 2004 (22–24). This change in RFLP typing pattern for ORF2 reflected a consistent change in the capsid gene sequence recognized by the 2 restriction enzymes *Xba*I and *Sma*I. This was supported by ORF2 gene sequencing at the time of the outbreak, with RFLP type 321 viruses having 99% sequence homology with each other, 91.6% homology with the previously dominant

Table IV. Probability values (PV), odds ratios (OR), 95% confidence intervals (CI) for the frequency of specific histological lesions that were significantly increased ($P < 0.05$) in pigs infected with the newer PCV-2 RFLP type 321 variant, versus the older RFLP type 422 strain

Histological lesion	PV	OR	95% CI
Lymph node — depletion of lymphoid follicles	0.0009	9.33	2.5, 34.58
Lymph node — histiocytic infiltration	0.0065	6.00	1.69, 21.26
Lymph node — giant cells	0.0037	6.90	1.84, 25.96
Spleen — histiocytic infiltration	0.0230	4.22	1.27, 14.09
Lung — interlobular edema	0.0041	6.75	1.83, 24.93
Lung — subpleural edema	0.0119	7.65	1.51, 38.76
Small intestine — infiltration lamina propria by histiocytes and lymphocytes	0.0093	5.88	1.60, 21.64
Colon — lamina propria lymphohistiocytic infiltration	0.0260	4.00	1.22, 13.14
Kidney — multifocal cortical interstitial aggregates of histiocytes and lymphocytes	0.0160	6.82	1.54, 30.15

RFLP 422 type, and 92% to 93% homology to those reported in the United States prior to 2005. Since 2005, this new variant virus has spread rapidly, becoming the dominant virus in Ontario and Quebec swine herds; it can be found across Canada (26). Sporadic cases of acute onset disease with similar high mortality in growing pigs have subsequently been reported from the USA (North Carolina, Kansas, and Iowa) (29), with similar devastating losses to the swine industry.

The retrospective comparison of full genome sequences (Figure 2) also shows the genetic variants with RFLP type 321 to be very different to the RFLP type 422 viruses, and similar to the FRA1-1998, FRA3-2000, Fd1-2004, Fd2-2004, and Fd3-2004 viruses found previously in France (30), with predicted RFLP type 321 and 99.3% sequence homology with the new variant RFLP type 321 Canadian viruses. Pigs were imported from France to Quebec immediately prior to the recognition of this genetic variant in Canada (Gagnon, personal communication). It may be that these imported pigs brought the PCV-2 variant from France into Quebec, with subsequent distribution to Ontario swine herds, due to the regular exchange of pigs between the 2 provinces. Unlike this report, however, studies in France concluded that their outbreaks were most likely not due to the emergence of a new variant (30). A previous genetic study of Canadian PCV-2 strains was unable to demonstrate relationships between genetic type and virulence (31); however, this earlier study was reported in 2002, before the introduction of these RFLP type 321 strains into Canada.

The PCV-2 viruses recently have been classified as PCV-2 group 1 or PCV-2 group 2 (12,29). A more recent Canadian report has subdivided PCV-2 strains as PCV-2a and PCV-2b (26). The newer variant PCV-2 RFLP type 321 viruses dominant in Ontario pigs since 2005 are similar to the group of viruses recently described as PCV-2b (26), and previously described as PCV-2 group 1 (12), which includes strains from France, China, Austria, the Netherlands, and the United Kingdom. The older Ontario PCV-2 RFLP type 422 strains dominant in Ontario swine prior to 2005 are similar to PCV-2 group 2a viruses (26), also referred to as PCV-2 group 2 (12). These include strains from Spain, Germany, Taiwan, China, and earlier strains from Canada and the United States. It would be important in the future to standardize the terminology used to distinguish these 2 groups of viruses.

The statistically significant increase in the frequency of histologic lesions in multiple tissues in pigs spontaneously infected with the new variant RFLP type 321, compared with the previously dominant RFLP type 422, as demonstrated by the probability values and odds ratios, implies increased virulence of this new strain. Others have also shown significant differences in microscopic lesions in lymphoid tissues in pigs experimentally infected with different strains of PCV-2 virus (27).

Increased viral burden, based on statistically higher IHC staining scores in lymph nodes for pigs infected with the newer variant RFLP type 321, compared to the older RFLP type 422, supports the suggestion of increased virulence associated with the new strain. Others have shown that viral burden in PCV-2 infected tissues, including lymphoid tissues, directly correlates with severity of clinical disease (32).

To the best of our knowledge, this is the first occurrence of splenic infarction identified in PCV-2 infected pigs, which was previously reported in brief (24). The recognition of this new lesion also supports the suggestion of increased virulence associated with RFLP type 321 strains. Granulomatous enteritis, necrotizing lymphadenitis and vasculitis have been reported in PCVAD syndromes (3).

Although dual infections with other pathogens may contribute to the severity of PCV-2 infection, the increase in virulence in pigs infected with this PCV-2 RFLP type 321 variant may be related to the genetic properties of the new virus, with corresponding changes in amino acids or other genetic changes such as silent mutation, which may affect transcription of viral mRNA, translation of viral proteins, and viral replication. This hypothesis is supported by the retrospective study with RFLP type 321 infected pigs having more frequent histologic lesions and significantly more viral antigen in lymph nodes, and the temporal association of more severe PCV-2 outbreaks as acknowledged by the Ontario swine industry where only previous RFLP type 422 strains had been endemic. This hypothesis is also supported by a recent study in which nucleotide changes at positions 1486–1472 in the capsid gene sequence of similar newly identified strains in the United States were reported to have changes in amino acids (29). This theory is again supported by studies in cell culture (33) where 2 amino acid mutations in the capsid protein of PCV-2 enhanced PCV-2 replication in vitro, while attenuating the

virus in vivo. A single amino acid mutation in the capsid protein of chicken anemia virus has also been identified as significant for pathogenicity in chickens (34). In addition, genetic differences in 2 highly characterized PCV-2 strains have been shown to directly affect virulence of PCV-2 in pigs (27) and it has been suggested that a range of virulence exits due to the genetic variation of PCV-2 strains (27). It will be important to test this hypothesis, comparing the new more virulent PCV-2 RFLP type 321 virus strains with earlier RFLP type 422 strains in controlled experimental challenge studies.

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